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ACETYLCHOLINESTERASE ACTIVITY IN ESERINE-TREATED ASCIDIAN EMBRYOS

DAVID FROMSON¹ AND J. R. WHITTAKER²

Department of Zoology, University of California, Los Angeles, California

Durante (1956, 1957) has shown histochemically that a cholinesterase is localized in the two lateral bands of muscle cells extending posteriorly in the larval tail of the ascidian, *Ciona intestinalis*. Since there was inhibition of normal muscle function in *Ciona* embryos and larvae exposed to eserine sulfate and other inhibitors of cholinesterase activity, the enzyme undoubtedly has a function in tail muscle contraction (Durante, 1958).

When *Ciona* embryos were treated with eserine during early embryonic stages, the inhibition of muscle activity which it caused was not reversible when the embryos were removed from the eserine solution, whereas, the inhibitory effect of eserine on fully developed tadpoles was reversible (Durante, 1958). Durante concluded that eserine may stop the synthesis of cholinesterase during development. The most interesting of the experiments she reports is a prefertilization effect of eserine. *Ciona* eggs placed in eserine for several hours prior to fertilization and subsequently fertilized and permitted to develop in eserine-free sea water, produced morphologically normal larvae which were quiescent, and which lacked sustained movements of the tail (Durante, 1958). This work supports the intriguing possibility that a specific enzyme inhibitor might prevent synthesis of the enzyme it inhibits.

The most plausible alternative to the explanation suggested by Durante is that eserine remains trapped in the egg or embryo, and that this residual eserine inhibits the muscular movement of the larva. However, it was the prefertilization experiment which led Reverberi (1961) to accept the conclusion that cholinesterase synthesis had been interfered with, because he considered it unlikely that the *Ciona* embryos could have retained eserine for such a relatively long time in pure sea water.

¹ Present address: Department of Biology, McGill University, Montreal 110, P.Q. Canada.

² Present address: The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104.

In view of the potential importance of the Durante results, we have undertaken to repeat the prefertilization experiment and to examine the possibility of eserine retention. Our work unequivocally shows that eserine is trapped in the egg in sufficient quantity to inhibit most of the acetylcholinesterase activity in the developing embryo, and that acetylcholinesterase synthesis is not affected by the drug.

MATERIALS AND METHODS

Animals

Adult specimens of *Ciona intestinalis* L. were collected at Marina del Rey, California, and gametes were obtained surgically as described by Costello, Davidson, Eggers, Fox and Henley (1957). The embryos were reared in filtered sea water and at a constant temperature of $18^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

Chemicals

Reagent grade chemicals were used throughout these studies. Eserine sulfate (H_2SO_4) USP was purchased from Calbiochem, BW62C47 [1:5-bis-(4-trimethyl-ammoniumphenyl) pentan-3-one diiodide] was a gift from the Burroughs-Wellcome Company, Tuckahoe, New York, and iso-OMPA (tetraisopropylpyrophosphoramidate) was purchased from L. Light and Company Ltd., Colnbrook, England. Dithiobisnitrobenzoic acid was purchased from Aldrich Chemical Company; acetylthiocholine iodide and butyrylthiocholine iodide were obtained from Nutritional Biochemicals Corporation.

Preparation of homogenates for enzyme assay

Embryos of the desired stage were washed with filtered sea water, and collected by low speed centrifugation. One to five thousand embryos were homogenized in a variable speed motorized Potter-Elvehjem homogenizer with a teflon pestle in a medium consisting of 0.1 M sodium phosphate buffer (pH 8.0) with 0.1% Triton X-100 (Rohm and Haas, Philadelphia, Pennsylvania). Homogenates were centrifuged in an International Centrifuge Model V at $750 \times g$ for 10 minutes, and the resulting supernatants assayed for acetylcholinesterase activity. Different homogenate supernatants contained 0.1–0.5 mg protein/ml.

Assay for acetylcholinesterase activity

A slightly modified method of Ellman, Courtney, Andres and Featherstone, (1961) was used to measure the acetylcholinesterase activity of homogenate supernatants (Fromson, 1968). Reaction mixtures contained 2.6 ml of 0.1 M sodium phosphate buffer (pH 8.0), 100 μl 0.1 M dithiobisnitrobenzoic acid in 0.1 M sodium phosphate buffer (pH 7.0), 100 μl 0.75 M acetylthiocholine iodide and 100 μl enzyme homogenate supernatant. The final substrate concentration in the reaction mixture was 2.5×10^{-2} M. This concentration was assumed to saturate the enzyme reaction based on the findings of Ellman *et al.* (1961). The reaction mixtures were incubated at 20°C in a water bath. Under these assay conditions,

Ciona enzyme activity was directly proportional to incubation time and directly proportional to the amount of homogenate protein in the reaction mixture.

The enzyme reaction velocity studied was the change in optical density (Δ O.D.) at 412 nm in a Zeiss PMQII spectrophotometer. In Table I, where per cent activities of the enzyme were being compared, enzyme activity was expressed as Δ O.D. 412 nm $\times 10^3$ /min. Otherwise, In Table II and Figure 1, enzyme activity was expressed as a specific activity, millimicromoles substrate hydrolyzed/min/mg protein, since results with different homogenates were being compared directly. Four activity measurements were made on each homogenate used and the results expressed as a mean $\pm$ the standard error (S.E.) of the mean.

Protein analysis

Protein was precipitated from samples of enzyme homogenate by the addition of trichloroacetic acid to a concentration of 5%. An acid-insoluble fraction was prepared according to Whittaker (1966). The Lowry method was used to measure total protein in these acid-insoluble fractions (Lowry, Rosebrough, Farr and Randall, 1951) with crystallized bovine serum albumin (Armour Pharmaceutical Company) as a quantitative standard.

Eserine treatment

Unfertilized eggs were removed surgically from the oviducts of adult specimens of *Ciona* and washed in several large volumes of filtered sea water. Eggs from each adult organism were kept separately at 18° C for 90–120 minutes (more time than required for the first cleavage to occur). The eggs were examined microscopically and only batches of non-dividing eggs were used. These unfertilized eggs were pooled and divided into two groups. One group was held as a control. The other group was treated with 0.002% (3×10^{-5} M) eserine sulfate for one hour, and washed free of drug with an excess of filtered sea water. Control and eserine-treated eggs were then fertilized by addition of a dilute sperm suspension, and the resulting embryos reared in eserine-free sea water to the tadpole larva stage (18–19 hours at 18° C). There was no evidence that fertilization occurred in the control and eserine-treated eggs prior to the addition of sperm suspension.

RESULTS

Nature of the cholinesterase enzyme

Since the measured cholinesterase activity could be caused by a pseudocholinesterase as well as a true acetylcholinesterase, two series of experiments were performed which would distinguish between these two enzymes. Activities were measured after treatment of the enzyme preparation with three specific enzyme inhibitors, and in the presence of the cholinesterase substrates acetyl- and butyrylthiocholine. The results of these experiments are presented in Table I.

The cholinesterase inhibitor eserine sulfate (Chadwick and Hill, 1947) was added to larva homogenates (1.5×10^{-5} M final concentration). After a 20–30 minute incubation at room temperature, no enzyme activity was detected in this

mixture. This rules out the possibility that a non-specific esterase is contributing to the cholinesterase reaction.

Larval cholinesterase was further characterized by the effect of specific enzyme inhibitors. Enzyme activity was measured in control homogenates, homogenates treated for 20 minutes with the pseudocholinesterase inhibitor iso-OMPA (tetra-isopropylpyrophosphoramidate, 10^{-3} M; Aldridge, 1953), and homogenates treated 20 minutes with the acetylcholinesterase inhibitor BW 62C47 [1:5-bis-(4-trimethylammoniumphenyl)pentan-3-one diiodide; Burgen, 1949]. The cholinesterase activity was inhibited 95.8% by BW 62C47 and only 5.5% by iso-OMPA. These data are consistent with the proposition that most of the enzyme activity is acetylcholinesterase activity.

TABLE I
Effect of substrates and specific enzyme inhibitors on the cholinesterase activity of embryo homogenates

Addition to reaction mixture	Function of additive	Enzyme activity ($\Delta 0.0412$ nm $\times 10^3$ /min) mean $\pm$ S.E.	Per cent activity
Homogenate 1			
Acetylthiocholine iodide (2.5×10^{-2} M)	Cholinesterase substrate	34.10 ± 0.36	100
Butyrylthiocholine iodide	Pseudocholinesterase substrate	1.53 ± 0.12	4.5
Homogenate 2*			
None (control)		16.58 ± 0.15	100
Iso-OMPA (10^{-3} M)	Pseudocholinesterase inhibitor	15.68 ± 0.12	94.5
BW62C47 (10^{-3} M)	Acetylcholinesterase inhibitor	0.70 ± 0.01	4.2
Eserine sulfate (1.5×10^{-3} M)	Cholinesterase inhibitor	0	0

* Acetylthiocholine iodide (2×10^{-2} M) used as substrate.

Acetylcholinesterase and pseudocholinesterase are also distinguished by their respective substrate specificities. Since acetylcholinesterase is highly specific for acetate containing substrates (Aldridge, 1953), enzyme activities were measured using the substrates acetyl- and butyrylthiocholine iodide. The measured enzyme activity with the butyrylthiocholine was only 4.5% of the activity measured using the acetylthiocholine iodide substrate. Therefore, the enzyme activity measured in tadpole homogenates is attributable to the presence of acetylcholinesterase.

Effects of eserine treatment

Unfertilized eggs were treated with 3×10^{-5} M eserine sulfate and washed free of the drug after one hour. These eggs were then fertilized and reared in eserine-free sea water. The resulting larvae exhibited a vastly reduced capacity for movement. Despite this striking physiological impairment, no morphological irregularities were observed upon microscopic examination of these larvae. These findings are similar to those reported by Durante (1958).

Acetylcholinesterase activity, measured in homogenates of larvae hatched from eserine-treated eggs (as described above), was inhibited 80–87% (Table II and

Fig. 1). These quantitative measurements of enzyme activity confirm Durante's (1958) hypothesis that embryos treated with eserine prior to fertilization are defective in acetylcholinesterase activity.

Eserine retention

Eserine accumulation and retention during the one hour prefertilization incubation is indicated by the following results. Acetylcholinesterase activity assayed in homogenates of larvae hatched from eserine-treated eggs was inhibited 80% (Table II). Homogenate mixtures containing equal amounts of control and eserine-treated embryo homogenates should have a predictable enzyme activity calculated by averaging the mean activity values for each of the two component homogenates.

TABLE II
Acetylcholinesterase activity of embryos treated prior to fertilization with eserine

Embryo homogenates	Acetylcholinesterase activity (μ moles substrate hydrolyzed/min. mg acid-insoluble protein) mean $\pm$ S.E.
Control	32.63 $\pm$ 0.57
Eserine-treated	6.60 $\pm$ 0.32
Calculated activity of a 1:1 mixture of control and eserine-treated embryo homogenates	19.61
Actual activity in a 1:1 mixture of control and eserine-treated embryo homogenates	6.23 $\pm$ 0.09

In the experiment reported in Table II, this calculated level was 56% of the control enzyme activity level. However, the actual acetylcholinesterase activity detected in the homogenate mixture was only 17% of the control level. Apparently, unfertilized *Ciona* eggs treated with eserine accumulate and retain this drug in sufficient quantities to inhibit the majority of the acetylcholinesterase activity in the homogenate mixture. In fact, the acetylcholinesterase activity in the mixed homogenate was not significantly different from the activity measured in the eserine-treated embryo homogenate alone.

Restoration of enzyme activity

If the sole action of the accumulated eserine is to inhibit enzyme activity, then the presence of this inhibitor during development should not interfere with acetylcholinesterase synthesis. Removal of the inhibitor from homogenates of prefertilization-treated embryos should restore acetylcholinesterase activity to the level of control homogenates. Eserine is a reversible inhibitor of acetylcholinesterase activity; it can easily be removed from the enzyme by dialysis (Cohen, Kalsbeek, and Warringa, 1948).

Homogenates were prepared from control and experimental larval stage embryos and acetylcholinesterase activity measured. The experimental embryos were treated with 3×10^{-5} M eserine sulfate for one hour prior to fertilization. In the experiment shown in Figure 1, eserine treatment resulted in an 87%

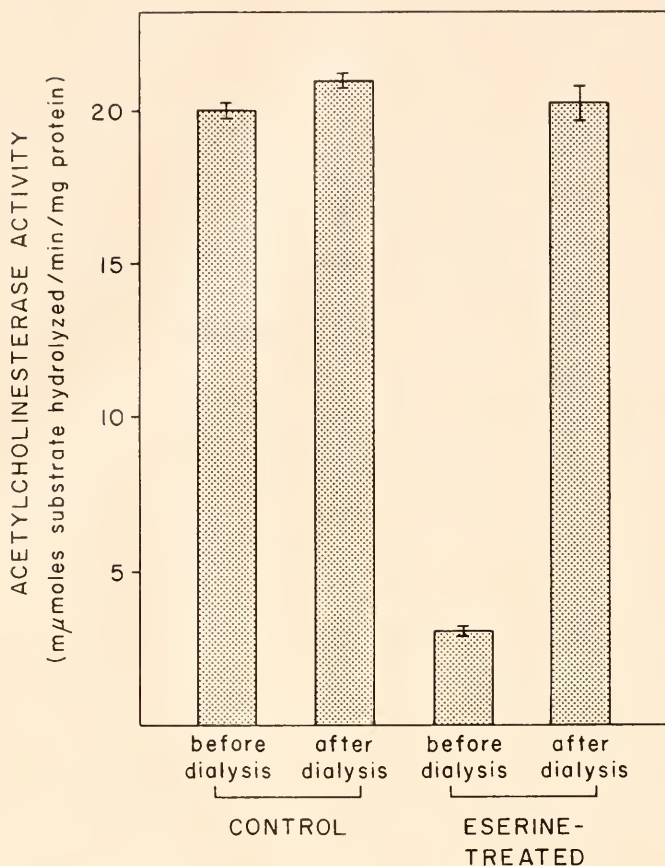


FIGURE 1. Unfertilized *Ciona* eggs incubated in $3 \times 10^{-5} M$ eserine sulfate for 1 hour prior to fertilization. The eggs were washed free of eserine, fertilized, and allowed to develop into larvae. Acetylcholinesterase was measured in control and eserine-treated larva homogenates. These two homogenates were then dialyzed against distilled water, and the post-dialysis enzyme activities of control and eserine-treated embryo homogenates measured. The bars represent the mean enzyme activity of 4 activity measurements on each homogenate; the vertical lines indicate the standard error of the mean.

inhibition of enzyme activity. These control and eserine-treated embryo homogenates were dialyzed against distilled water for 55 hours at room temperature. After dialysis, the enzyme activities in the homogenates were determined once again. Eserine inhibition was completely reversed by dialysis; acetylcholinesterase activities in the dialyzed control and the dialyzed experimental embryo homogenates were equal (Fig. 1). Since dialysis restores the level of enzyme activity in the experimental homogenate to that found in the control homogenate, the presence of eserine has obviously not prevented the synthesis of a full complement of acetylcholinesterase in these embryos.

An interesting point concerning the stability of *Ciona* acetylcholinesterase is illustrated by the dialysis of control homogenate in Figure 1. Since there is no loss of activity following a dialysis of two days duration at room temperature, the enzyme is shown to be remarkably resistant to denaturation. This is also true for acetylcholinesterase from other animal sources (Nachmansohn and Wilson, 1955).

DISCUSSION

Sawyer (1943) has shown clearly that eserine does not prevent synthesis of cholinesterase in developing *Amblystoma* embryos. Therefore, there is little reason to accept the conclusion that Durante (1958) reaches from her experiments with *Ciona* embryos, namely, that eserine has interfered with the synthesis of cholinesterase. Unlike Sawyer, Durante has no supporting evidence from enzyme studies; her conclusion is based solely on the irreversible inhibition of larval motility caused by eserine treatments. The prefertilization experiment with eserine provides the best circumstantial evidence that enzyme synthesis may be deficient, because, as Reverberi (1961) points out, eserine would have to be retained in the egg for an unusually long time if trapped eserine is the real cause of the inhibited motility.

We know of no evidence from any biological system that a specific enzyme inhibitor which is not also a substrate analog could possibly prevent synthesis of an enzyme. On further investigation we have discovered that the Durante work is no exception. There is enough eserine trapped in *Ciona* embryos following prefertilization treatment to inhibit the acetylcholinesterase of control embryos when homogenates of both are mixed together. Full acetylcholinesterase activity can be restored in homogenates of experimental embryos by simple dialysis. All of the Durante results can be explained by retention of eserine in the egg or embryo following even a brief exposure to the drug.

Based on the Durante (1958) experiments, eserine seems to be freely diffusible into the egg at any stage of development and retained by all the pre-hatching stages. Since reversal of the reduced mobility caused by eserine occurred only in larvae which were first treated with the drug after hatching, it seems likely that the egg membranes are a permeability barrier which restricts loss of eserine in eserine-free sea water. This question could be studied further using enzyme preparations which de-chorionate ascidian eggs (Berrill, 1937).

Our investigation also confirms an important observation made by both Sawyer (1943) and Durante (1958). Differentiation of the morphological structure as well as the potential physiological function of an acetylcholinesterase-containing tissue is independent of the functional activity of the enzyme during development. Likewise, the function of tyrosinase in ascidian pigment cells is not necessary for normal differentiation of the cells (Minganti, 1957; Whittaker, 1960, 1966). This principle probably applies to the specialized enzyme systems of most differentiating cells and tissues.

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SUMMARY

1. The use of specific cholinesterase inhibitors and substrates demonstrated that the enzyme activity in *Ciona intestinalis* larvae is an acetylcholinesterase.

2. Eggs treated with eserine sulfate (an acetylcholinesterase inhibitor) for one hour prior to fertilization developed into larvae with defective muscular movements and greatly reduced levels of acetylcholinesterase activity.

3. Two kinds of experiments show that this reduced enzyme activity was caused by the retention of eserine and not by inhibition of acetylcholinesterase synthesis. Homogenates of embryos from eserine-treated eggs inhibit acetylcholinesterase activity when mixed with homogenates of control embryos. Full enzyme activity in homogenates of the experimental embryos could be recovered by dialysis.

LITERATURE CITED

- ALDRIDGE, W. N., 1953. The differentiation of true and pseudocholinesterase by organophosphorus compounds. *Biochem. J.*, **53**: 62-67.
- BERRILL, N. J., 1937. Culture methods for ascidians. Pages 564-571 in: P. S. Galtsoff, F. E. Lutz, P. S. Welch and J. G. Needham, Eds., *Culture Methods for Invertebrate Animals*. Comstock, Ithaca.
- BURGEN, A. S. V., 1949. The mechanism of action of anticholinesterase drugs. *Brit. J. Pharmacol.*, **4**: 219-228.
- CHADWICK, L. E., AND D. HILL, 1947. Inhibition of cholinesterase by diisopropylfluorophosphate, physostigmine, and hexacthyl tetraphosphate in the roach. *J. Neurophysiol.*, **10**: 235-246.
- COIJEN, J. A., F. KALSBECK AND M. G. P. J. WARRINGA, 1948. Reversibility of the inhibition of true cholinesterase by physostigmine. *Biochim. Biophys. Acta*, **2**: 549-560.
- COSTELLO, D. P., M. E. DAVIDSON, A. EGGERS, M. H. FOX AND C. HENLEY, 1957. *Methods for Obtaining and Handling Marine Eggs and Embryos*. Marine Biological Laboratory, Woods Hole, Massachusetts, 247 pp.
- DURANTE, M., 1956. Cholinesterase in the development of the ascidian, *Ciona intestinalis*. *Experientia*, **12**: 307-308.
- DURANTE, M., 1957. Cholinesterase in the anterior and posterior hemiembryos of *Ciona intestinalis*. *Acta Embryol. Morphol. Exp.*, **1**: 131-133.
- DURANTE, M., 1958. Action of cholinesterase inhibitors on ascidian embryos. *Acta Embryol. Morphol. Exp.*, **1**: 273-279.
- ELLMAN, G. L., K. D. COURTNEY, V. ANDRES AND R. M. FEATHERSTONE, 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**: 88-95.
- FROMSON, D. R., 1968. Synthesis of acetylcholinesterase during embryogenesis of *Ciona intestinalis*. *Ph.D. thesis, University of California at Los Angeles*, 101 pp.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265-275.
- MINGANTI, A., 1957. Inhibition of melanogenesis in *Phallusia* embryos (Ascidians). *Acta Embryol. Morphol. Exp.*, **1**: 37-47.
- NACHMANSOHN, D., AND I. B. WILSON, 1955. Acetylcholinesterase. Pages 642-651 in S. P. Colowick and N. O. Kaplan, Eds., *Methods in Enzymology*, Vol. I. Academic Press, Inc., New York.

- REVERBERI, G., 1961. The embryology of ascidians. Pages 55-101 in M. Abercrombie and J. Brachet, Eds., *Advances in Morphogenesis, Volume 1*. Academic Press, Inc., New York.
- SAWYER, C. H., 1943. Cholinesterase and the behavior problem in *Amblystoma*. I. The relationship between the development of the enzyme and early motility. II. The effects of inhibiting cholinesterase. *J. Exp. Zool.*, **92**: 1-30.
- WHITTAKER, J. R., 1960. An *in vivo* analysis of tyrosinase function and melanin formation in ascidian embryos. *Anat. Rec.*, **138**: 388-389.
- WHITTAKER, J. R., 1966. An analysis of melanogenesis in differentiating pigment cells of ascidian embryos. *Develop. Biol.*, **14**: 1-39.